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APPLICATION NUMBER: ***60/394,199***
FILING DATE: ***July 05, 2002***
RELATED PCT APPLICATION NUMBER: *PCT/US03/21094*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

P. R. Grant

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PTO/SB/16 (10-01)
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV118434190US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Jian	Liu	Chapel Hill, NC
Guoqing	Xia	Hamberg, Germany
Jinghua	Chen	Chapel Hill, NC

[X] Additional inventors are being named on the 1 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

HEPARAN SULFATE 3-O-SULFOTRANSFERASE ISOFORM 5 GENERATES BOTH AN ANTITHROMBIN-BINDING SITE AND AN ENTRY RECEPTOR FOR HERPES SIMPLEX VIRUS-1

CORRESPONDENCE ADDRESS

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PATENT TRADEMARK OFFICE

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ENCLOSED APPLICATION PARTS (check all that apply)

[X] Specification	Number of Pages 38	[ ] CD(s), Number	
[ ] Drawing(s)	Number of Sheets	[ ] Other (specify)	
[ ] Application Data Sheet. See 37 CFR 1.76			

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

		FILING FEE AMOUNT ($)
[ ]	Applicant claims small entity status. See 37 CFR 1.27.	
[ ]	A check or money order is enclosed to cover the filing fees	
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[ ]	Payment by credit card. Form PTO-2038 is attached.	

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[ ] No

[X] Yes, the name of the U.S. Government agency and the Government contract number are: NIH AI50050-01 (to J.L.) AI053836-01 (to D.S.)

Respectfully submitted,

SIGNATURE Arles A. Taylor, Jr.

Date 07/05/2002

TYPED or PRINTED NAME Arles A. Taylor, Jr.

REGISTRATION NO. (if appropriate) 39,395

Docket Number: 421/67

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number	421/67

INVENTOR(S)/APPLICANT(S)

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Vaibhav	Tiwari	Chicago, IL
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Jin-Ping	Li	Uppsala, Sweden

Number 1 of 1

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FEE TRANSMITTAL for FY 2002

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☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 160.00

Complete if Known	
Application Number	
Filing Date	
First Named Inventor	Jian Liu
Examiner Name	
Group Art Unit	
Attorney Docket No.	421/67

METHOD OF PAYMENT *(check all that apply)*

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: 50-0426

Deposit Account Name: Jenkins & Wilson

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FEE CALCULATION

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101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	160.00

SUBTOTAL (1) ($) 160.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below	=	Fee Paid
Total Claims		-20** =		X	=
Independent Claims		- 3** =		X	=
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17(q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1 129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBMITTED BY				Complete *(if applicable)*	
Name (Print/Type)	Arles A. Taylor, Jr.	*Registration No (Attorney/Agent)*	39,395	*Telephone*	919-493-8000
Signature	Arles A. Taylor			*Date*	July 5, 2002

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60394199 .070502

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July 5, 2002

"Express Mail" mailing number: EV118434190US
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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C.

Patty Wilson

Commissioner for Patents
BOX PROVISIONAL APPLICATION
Washington, D.C. 20231

Re: U.S. Provisional Patent Application for HEPARAN SULFATE 3-O-SULFOTRANSFERASE ISOFORM 5 GENERATES BOTH AN ANTITHROMBIN-BINDING SITE AND AN ENTRY RECEPTOR FOR HERPES SIMPLEX VIRUS-1
Our File No. 421/67

Sir:

Please find enclosed the following:

1. A U.S. provisional patent application HEPARAN SULFATE 3-O-SULFOTRANSFERASE ISOFORM 5 GENERATES BOTH AN ANTITHROMBIN-BINDING SITE AND AN ENTRY RECEPTOR FOR HERPES SIMPLEX VIRUS-1 (38 pages);

2. Provisional Application for Patent Cover Sheet (Form PTO/SB/16) in duplicate (2 pages);

3. Fee Transmittal (Form PTO/SB/17) in duplicate;

4. A return-receipt postcard to be returned to our offices with the U.S. Patent and Trademark Office date stamp thereon; and

5. A Certificate of Express Mail No.: EV118434190US

Please contact our offices if there are any questions.

The Commissioner is hereby authorized to charge any deficiency or credit any overpayment associated with the filing of this correspondence to Deposit Account Number **50-0426**.

Respectfully submitted,

JENKINS & WILSON, P.A.

Arles A. Taylor, Jr.
Registration No. 39,395
Customer No. Bar Code Label: 25297
PATENT TRADEMARK OFFICE

AAT/ptw

Enclosures

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Date of Deposit 5 July 2002
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Patty Wilson
Patty Wilson

Description

Heparan Sulfate 3-*O*-sulfotransferase Isoform 5 Generates both an Antithrombin-binding Site and an Entry Receptor for Herpes Simplex Virus-1

Statements of the Invention

We have identified a gene that can synthesize both anticoagulant heparan sulfate and an enti receptor for herpes simplex virus.

Cell surface heparan sulfate, a highly sulfated polysaccharide, can interact with protease inhib to prevent blood from clotting (anticoagulant heparan sulfate). In addition, another subtype of heparan sulfate can be recognized by herpes simplex virus as an entry receptor for its infectio Both anticoagulant heparan sulfate and the heparan sulfate for herpes simplex viral receptors contain unique sulfated saccharide sequences. Those sequences are synthesized by heparai sulfate 3-O-sulfotransferase. In this invention, we discovered a heparan sulfate 3-O-sulfotransferase isoform synthesizes both anticoagulant heparan sulfate and herpes simplex v receptor. This gene is a new target for drugs to treat diseases that are related to anticoagulation and herpes simplex viral infection.

The previous discovery found that the enzymes for generating anticoagulant heparan sulfate herpes simplex viral receptor are distinct. Here, we found that a new isoform of 3-O-sulfotransferase, 3-OST-5, is capable of generating those two biologically active heparan sulf In addition, 3-OST-5 enzyme is predominantly expressed in human skeletal muscle tissue.

This is particularly important for human gene therapy in skeletal muscle to treat duchenne muscular dystrophy using herpes simplex virus 1 as a viral vector. Our invention reveals a potential gene that synthesizes the viral receptor for this therapy.

Heparan Sulfate 3-*O*-sulfotransferase Isoform 5 Generates both an Antithrombin-binding Site and an Entry Receptor for Herpes Simplex Virus-1

Guoqing Xia[1], Jinghua Chen[2], Vaibhav Tiwari[3], Wujian Ju[2], Jin-Ping Li[4], Anders Malmström[1], Deepak Shukla[3], Jian Liu[2¶]

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The nucleotide sequence reported in this paper has been submitted to the GenBank™/ EBI Data Bank with accession number AF503292

¶ To whom correspondence and reprint requests should be addressed: Rm 350A, Taylor Hall, CB#7090, University of North Carolina, Chapel Hill, NC 27599. Tel: (919)-843-6511; FAX: (919)-843-5432; email: jian_liu@unc.edu.

This work is supported by National Institutes of Health grants AI50050-01 (to J.L.), AI053836-01 (to D.S.), a grant from Pharmacy Foundation of North Carolina (to J.L.) and a grant from Swedish Research Council 7479 (to A.M.).

Running title: "Heparan sulfate 3-*O*-sulfotransferase"

Abstract

Heparan sulfate 3-*O*-sulfotransferase transfers sulfate to the 3-OH position of a glucosamine residue of heparan sulfate (HS) to form 3-*O*-sulfated HS. The 3-*O*-sulfated glucosamine residue contributes to two important biological functions of HS: binding to antithrombin thereby carrying anticoagulant activity and binding to herpes simplex viral envelope glycoprotein D (gD) to serve as an entry receptor for herpes simplex virus 1. A total of five HS 3-*O*-sulfotransferase isoforms were previously reported. Here we report the isolation and characterization of a novel HS 3-*O*-sulfotransferase isoform, designated as HS 3-*O*-sulfotransferase isoform 5 (3-OST-5). 3-OST-5 cDNA was isolated from a human placenta cDNA library and expressed in COS-7 cells. The disaccharide analysis of 3-OST-5 modified HS revealed that 3-OST-5 generated at least three 3-*O*-sulfated disaccharides: IdoUA2S-AnMan3S, GlcUA-AnMan3S6S, and IdoUA2S-AnMan3S6S. Transfection of the plasmid expressing 3-OST-5 rendered wild type CHO cells susceptible to the infection by herpes simplex virus 1, suggesting that 3-OST-5 modified HS serves as an entry receptor for herpes simplex virus 1. In addition, 3-OST-5 modified HS bound to herpes simplex viral envelope protein gD. Furthermore, we found that 3-OST-5 modified HS also bound to antithrombin, suggesting that 3-OST-5 also produces anticoagulant HS. In summary, our results indicate that a new member of 3-OST family generates both anticoagulant HS and an entry receptor for herpes simplex virus 1. These results provide a new insight regarding the mechanism for the biosynthesis of biologically active HS.

Introduction

Heparan sulfates (HSs) are highly sulfated polysaccharides, present on the surface of mammalian cells and in the extracellular matrix in large quantities. HSs play critical roles in a variety of important biological processes, including assisting viral infection, regulating blood coagulation and embryonic development, suppressing tumor growth and controlling the eating behavior of mice by interacting with specific regulatory proteins (1-5). HS polysaccharides carry negative charges under physiological pH, and the disaccharide repeating units consist of 1→4-linked sulfated glucosamine and uronic acid. The unique sequences determine to which specific proteins HS bind, thereby regulating biological processes.

The biosynthesis of HS occurs in the Golgi apparatus. It is initially synthesized as a copolymer of glucuronic acid and *N*-acetylated glucosamine by D-glucuronyl and *N*-acetyl-D-glucosaminyl transferase, followed by various modifications (6). These modifications include *N*-deacetylation and *N*-sulfation of glucosamine, C_5-epimerization of glucuronic acid to form iduronic acid residues, 2-*O*-sulfation of iduronic and glucuronic acid, as well as 6-*O*-sulfation and 3-*O*-sulfation of glucosamine. Several enzymes that are responsible for the biosynthesis of HS have been cloned and characterized (see review by Esko and Lindahl) (7). These enzymes have become essential tools for investigating the relationship between the structures and functions of HS.

What is still unknown is the detailed mechanism for regulating the biosynthesis of HS with a defined saccharide sequence. A recent report suggests that the expression levels of various HS biosynthetic enzyme isoforms contribute to the synthesis of specific

saccharide sequences in specific tissues (8). HS *N*-deacetylase/*N*-sulfotransferase, 3-*O*-sulfotransferase, and 6-*O*-sulfotransferase are present in multiple isoforms. Each isoform is believed to recognize a saccharide sequence around the modification site in order to generate a specific sulfated saccharide sequence (8-10). For instance, HS D-glucosaminyl 3-*O*-sulfotransferase (3-OST) isoforms generate 3-*O*-sulfated glucosamine residues that are linked to different sulfated uronic acid residues. 3-OST-1 transfers sulfate to the 3-OH position of an *N*-sulfated glucosamine residue that is linked to a glucuronic acid residue at the nonreducing end (GlcUA-GlcNS±6S). However, 3-OST-3 transfers sulfate to the 3-OH position of an *N*-unsubstituted glucosamine residue that is linked to a 2-*O*-sulfated iduronic acid at the nonreducing end (IdoUA2S-GlcNH$_2$±6S) (11). The difference in the substrate specificity of 3-OSTs results in distinct biological functions. For example, the HS modified by 3-OST-1 binds to antithrombin (AT) and has anticoagulant activity (12). However, the HS modified by 3-OST-3 (3-OST-3A and 3-OST-3B)[1] binds to glycoprotein D (gD) of herpes simplex virus-1, thus mediating viral entry (13).

The HS- and heparin-regulated anticoagulation mechanism has been studied extensively. It is now known that HS and heparin interact with AT, a serine protease inhibitor, to inhibit the activities of thrombin and factor Xa in the blood coagulation cascade (2). Anticoagulant-active HS (HS^{act}) and heparin contain one or multiple AT binding sites per polysaccharide chain . This binding site contains a specific pentasaccharide sequence with a structure of –GlcNS(or Ac)6S-GlcUA-GlcNS3S(±6S)-IdoUA2S-GlcNS6S-. The 3-*O*-sulfation of glucosamine for generating GlcNS3S(±6S)

residue, which is carried out by 3-OST-1 (EC 2.8.2.23), is the critical modification for the synthesis of HS^{act} (12,14).

Cell surface HS assists herpes simplex viral infection (15). A recent report suggests that a specific 3-*O*-sulfated HS is involved in assisting HSV-1 entry (13). The 3-*O*-sulfated HS is generated by 3-OST-3, but not by 3-OST-1. In addition, the 3-*O*-sulfated HS provides binding sites for HSV-1 envelope glycoprotein gD, which is a key viral protein involved in the entry of HSV-1(13). Since 3-OST-3 modified HS is rarely found in HS from natural sources, the study suggests that HSV-1 recognizes a unique saccharide structure. Indeed, the result from the structural characterization of a gD-binding octasaccharide revealed that the octasaccharide possesses a specific saccharide sequence (16). In addition, the binding affinity of the 3-*O*-sulfated HS for gD is about 2 μM (13). This affinity is similar to that reported for the binding of gD to the protein receptors, suggesting that HSV-1 utilizes both protein and HS cell surface receptors to infect target cells (17,18). It is believed that the interaction between gD and the 3-*O*-sulfated HS or the protein entry receptors somehow triggers the fusion between the virus and the cell in the presence of other viral envelope proteins, including gB, gH and gL (19). A study of the cocrystal structure of gD and herpes entry receptor HveA suggests that the binding of HveA to gD induces conformational changes in gD (20).

It is known that the enzymes for synthesizing HS^{act} and an entry receptor for HSV-1 belong to two 3-OST isoforms. These two isoforms generate the 3-*O*-sulfated glucosamine residue located in different saccharide sequences as described above. In this manuscript, we report that a 3-OST homologous protein, designated as HS 3-*O*-sulfotransferase isoform-5 (3-OST-5), possesses the activities for generating both HS^{act}

and HSV-1 entry receptor (or gD-binding HS). Our results suggest that the biosynthesis of HS^{act} and the gD-binding HS is regulated by several enzymes. The newly identified enzyme provides an additional tool for both understanding the mechanism for the biosynthesis of the biologically active HS and for investigating the relationship between the saccharide sequences and the biological functions of HS.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human 3-OST-3A and mouse 3-OST-1 enzymes were expressed in SF9 cells using baculovirus expression system. The enzymes were purified by heparin-toyopearl and 3',5'-ADP-agarose chromatographies as described previously (11,21). [^{35}S]PAPS was prepared by incubating 0.4 to 2 mCi/mL [^{35}S]Na_2SO_4 (carrier-free, ICN) and 16 mM ATP with 5 mg/mL dialyzed yeast extract (Sigma) (12). HS was either isolated from 33-cells, a L-cells variant, or from Chinese hamster ovary (CHO) cells as described previously (12). The concentrations of the unlabeled HS were determined by a method reported by Björnsson (22). Preparation of metabolically ^{35}S-labeled HS from CHO cells was described elsewhere (23). Human AT is from Cutter Biological (Berkeley, CA). A truncated form of herpes simplex virus 1 glycoprotein D, gD-1(306t), and monoclonal anti-gD (DL6) were generous gifts of Drs. Cohen and Eisenberg of University of Pennsylvania (24). The ^{3}H-labeled disaccharide standards, GlcUA-AnMan3S6S and IdoUA2S-AnMan6S, were prepared from ^{3}H-labeled HS (gifts from Dr. Rosenberg, Massachusetts Institute of Technology) (25). The ^{35}S-labeled disaccharide standards, IdoUA2S-AnMan3S and IdoUA2S-AnMan3S6S, were prepared from low-pH

(pH 1.5) nitrous acid degraded HS that was modified by purified 3-OST-3 enzyme as described by Liu *et. al.* (11).

Isolation of 3-OST-5 cDNA

GeneBank database was probed with the amino acid sequence of 3-OST-1 using tBlastn. By using Genscan, we predicted a 1,041 bp open reading frame that encodes a homologous protein from a genomic clone RP11-112L15 with GenBank™ accession number AL355498. The open reading frame sequence, designated as 3-OST-5, was located in two exons. Both 5'- and 3'-primers were synthesized based upon the sequence of Exon 1 and Exon 2, respectively. The following are the sequences of the two specific primers: 5'- GGAGGGCC ATG CTA TTC AAA CAG-3' (5`-primer), and 5'-TTA GGG CCA GTT CAA TGT CCT -3` (3'-primer). We cloned the cDNA using PCR from a human placenta cDNA library with the two specific primers. The resultant PCR product (about 1.0 Kb) was inserted into the pGEM-T-easy vector (pGEM-T-3OST5), and sequenced for both strands. The isolated cDNA has an identical sequence to which was predicted from the genomic clone. 5'-rapid amplification of cDNA ends (5'-RACE) was performed but failed.

Cell culture

Both COS-7 and wild type CHO cells were from Dr. R. Jude Samulski (University of North Carolina). COS-7 cells and CHO cells were maintained in logarithmic growth by subculturing biweekly at 37°C under 6% CO_2 humidified atmosphere. COS-7 cells and CHO cells were grown in Dulbecco's modified Eagle's medium (Gibco) and in F12 medium (Gibco) supplemented with 10% fetal bovine serum (JRH), respectively.

Expression of 3-OST-5

3-OST-5 expression plasmid was constructed by inserting the open reading frame of 3-OST-5 into a pcDNA3.1 (Invitrogen) expression vector. The open reading frame was obtained by reamplifying the sequence from pGEM-T-3OST5 by PCR using the following primers: 1) the 5`-specific primer, 5'-TCA AAGCTT *GCCACC* ATG CTATTCAAACAGCA-3', contains a *Hind* III site (underlined), the consensus Kozak sequence (italicized) and a start codon; and 2) the 3`-specific primer, 5'- GC TCTAGA TTAGGGCCAGTTCAATGTCCT-3`, contains an *Xba* I site (underlined). PCR reactions were carried out using Advantage-2 PCR kit (Clontech, USA) with the initial denaturation for 2 min at 94°C, followed by 30 cycles of a reaction consisting of 45 s for denaturation at 94 °C, 45 s for annealing at 62°C, and 90 s for elongation at 72°C. The PCR fragment was subcloned into pcDNA3.1 (Invitrogen) using *Hind* III/*Xba* I sites. The coding region of the construct was completely sequenced on both strands, and the construct was designated pcDNA3.1-3OST5. pcDNA3.1-3OST5 or pcDNA3 plasmid was transfected into exponentially growing COS-7 cells using Lipofectamine 2000 (Gibco).

Measurement of 3-OST-5 activity

Preparation of [^{35}S]HS-The crude enzyme was extracted from transfected COS-7 cells. The cells were harvested 72 hours after transfection. Approximately 3 x 10^6 cells were mixed with 100 µL of cold 0.25 M sucrose containing 1% Triton X-100 (v/v) and incubated on ice for 30 min. The insoluble residues were removed after centrifuging at 10,000 x *g* for 10 min. The HS sulfotransferase activity was determined by incubating 40 µg of cell extract with 1 µg of unlabeled HS (from 33-cells), 4 x 10^7 cpm of [^{35}S]PAPS,

in 50 μL of a buffer containing 50 mM 2-[*N*-morphilino]ethanesulfonic acid, 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 1% Triton X-100, pH 7. The reaction was incubated at 37°C for 2 h, quenched by heating at 100°C for 1 min and centrifuged at 10,000 x *g* for 1 min to remove insoluble materials. The sample was then subjected to a 200-μL DEAE-Sepharose column to purify [^{35}S]HS (12).

Disaccharide analysis of [^{35}S]HS-The [^{35}S]HS modified by 3-OST-5 was mixed with 20 μg of unlabeled HS (from ICN) and degraded with nitrous acid at pH 1.5 followed by reduction with sodium borohydride (26). The resultant ^{35}S-labeled disaccharides were mixed with a ^{3}H-labeled disaccharide standard, IdoUA2S-[1-^{3}H]AnMan6S, and desalted on a BioGel P-2 column (0.75 x 200 cm) which was equilibrated with 0.1 M ammonium bicarbonate at a flow rate of 4 mL/h. The ^{3}H-labeled disaccharide standard was used to locate the elution position of the disaccharides from the BioGel P-2 column.

The disaccharides were resolved by a C_{18}-reversed phase column (0.46 x 25 cm) (Vydac) under the reverse-phase ion-pairing HPLC (RPIP-HPLC) condition (11). Briefly, the column was eluted with acetonitrile as follows: 8% for 30 min followed by 15% for 15 min and followed by 19.5%, in a solution containing 38 mM ammonium phosphate monobasic, 2 mM phosphoric acid, and 1 mM tetrabutylammonium phosphate monobasic (Fluka) at a flow rate of 0.5 mL/min.

Characterization of 3-OST-5 modified HS

Preparation of 3-OST-1 and 3-OST-3 modified HS. To prepare 3-OST-3 modified HS, HS (from 33-cells, 1 μg) was mixed with 30 ng of purified 3-OST-3A enzyme and [^{35}S]PAPS (4 x 10^7 cpm) in a buffer containing 50 mM 2-[*N*-morphilino]ethanesulfonic acid, 1% Triton X-100, 1 mM $MgCl_2$, 2 mM $MnCl_2$, 150 mM NaCl and 168 μg/mL

bovine serum albumin, pH 7, in a final volume of 50 μL. The reaction was incubated at 37°C for 2 h and was then heated at 100°C for 2 min. The resultant was centrifuged at 14,000 rpm for 1 min to remove insoluble materials. The supernatant was loaded onto a 200 μL-DEAE-Sepharose column, and the [^{35}S]HS was eluted from the column with 1000 mM NaCl (12). To prepare 3-OST-1 modified HS, we utilized 70 ng of purified enzyme and followed nearly identical procedures except for omitting the 150 mM NaCl.

Demonstration of 3-OST-5 modified HS binding to AT. The binding of the 3-OST-5 modified HS to AT was determined using an AT/Concanavalin A (ConA)-Sepharose approach (12). Briefly, HS (10,000 to 100,000 cpm) was incubated in 150 μL of a buffer, which contains10 mM Tris-HCl, 150 mM NaCl, 1 μM dextran sulfate, 1 mM Ca^{2+}, Mg^{2+} and Mn^{2+}, and 0.1 mg/mL of AT (pH 7.5), at room temperature for 30 min. The solution was mixed with the prewashed ConA-Sepharose (60 μL of 1:1 slurry) and agitated at room temperature for one hour. The gel was then washed with 3 x 1 mL of a buffer containing 10 mM Tris-HCl, 0.0004% Triton X-100 and 150 mM NaCl (pH 7.5). The HS was eluted from the gel by 1 mL of a buffer containing 10 mM Tris-HCl, 1000 mM NaCl, and 0.0004% Triton X-100 (pH 7.5).

Determination of the binding of 3-OST-5 modified HS to gD. The assay for determining the binding of 3-*O*-sulfated HS to gD was carried out by an immunoprecipitation procedure using anti-gD monoclonal antibody (13). The enzyme modified HS (100,000 to 200,000 cpm) was incubated in 50 μL of a buffer containing 50 mM Tris-HCl, 150 mM NaCl and 0.01% Triton, pH 7 (binding buffer) and 2 mg/mL of gD at room temperature for 30 min. The anti-gD monoclonal antibody DL6 (5 μL) was added and incubated at 4°C for 1 h followed by the addition of protein A-Agarose gel (80 μL of 1:1

slurry) and agitated at 4°C for an additional hour. The HS was eluted from the gel with 1 mL of 1000 mM NaCl in the binding buffer.

Determination of HS^{act} conversion activity. The assay was specifically designed to determine the HS biosynthetic activity that generates HS^{act} (12). Briefly, cell extract was incubated with metabolically ^{35}S-labeled nonanticoagulant HS (from wild type CHO cells) and unlabeled PAPS. The resultant [^{35}S]HS was subjected to the AT-binding assay as described above. The increase in the percentage of the [^{35}S]HS that binds to AT correlated to the amount of HS^{act} conversion activity in the cell extract.

Herpes simplex viral entry assay

The conditions for growing cells and different HSV strains were described previously (13). The viral infectivity assay is based on visualization of the cells carrying β-galactosidase activity (25). CHO cells were transfected in six-well dishes, using Lipofectamine (Gibco) with pcDNA3.1-3OST5 plasmid or control plasmid (pcDNA3) at 1.5 to 2.0 μg per well in 1 mL. At about 36 h post-transfection, cells were exposed to recombinant HSV-1 [HSV-1(KOS) gL86] (gift from Dr. Spear, Northwestern University) that expresses β-galactosidase upon viral entry. At 6 h post-infection, the cells were fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde, permeabilized in 2 mM $MgCl_2$ containing 0.01% deoxycholate and 0.02% NP-40, and incubated with buffered X-gal (0.5 mg/mL). Three hours later the infected cells were visible in blue due to the action of β-galactosidase on X-gal. The transfection efficiency for CHO cells was determined by transfecting a plasmid expressing β-galactosidase as a reporter gene.

Northern blot analysis

The coding sequence of 3-OST-5 was labeled with [γ-^{32}P]dCTP in a reaction with Klenow enzyme (Roche), and used as a probe to hybridize the Human Multiple Tissue Northern (MTN®) blot (Clontech). The hybridization was carried out in ExpressHyb Hybridization Solution (Clontech) at 60°C for one hour, and the blot was washed with 0.1 x SSC containing 0.5% SDS at 60°C for 40 min (where 1 x SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0). The membrane was exposed to an X-ray film for 4 days.

RESULTS

Isolation of the cDNA encoding 3-OST-5

Probing the non-redundant database of National Centre for Biotechnology Information with the deduced amino acid sequence of human 3-OST-1 (accession no. AF033827), we identified a 165 Kb genomic clone with GenBank™ accession number AL355498. An open reading frame was found to be 1041 bp. The predicted open reading frame was located in two exons that were gapped by a 4.5-Kb intron. The open reading frame of this protein, assigned as 3-OST-5, was amplified from a human placenta cDNA library using specific 5'- and 3'-primers as described under "Experimental Procedures". 3-OST-5 cDNA sequence and the predicted amino acid sequence are shown in Figure 1. We attempted to obtain further upstream of 3-OST-5 sequence using 5'-RACE but failed. The isolated 3-OST-5 cDNA contains 1041 bp and the deduced peptide of 346 amino acid residues predicts a type II membrane-bound protein. The protein has four potential *N*-glycosylation sites with the predicted molecular weight of 40,407 Da. The deduced amino acid sequence of 3-OST-5 has 71 % and 58 % homology to 3-OST-1 and 3-OST-3 in the sulfotransferase domains, respectively (Figure 2). Putative PAPS binding sites

were also found in 3-OST-5 based upon the PAPS-binding consensus sequences (27,28) (Figure 2). The genomic BAC clone RP11-112L15, which contains 3-OST-5 gene, was annotated to be mapped on human chromosome 11q22.2. Alignment of the genomic sequence with 3-OST-5 cDNA revealed that Exon 1 and Exon 2 contain 107 bp and 934 bp of the open reading frame, respectively.

Determination of the [^{35}S]sulfation site of 3-OST-5 modified HS

Because the cloned 3-OST-5 has high homology to 3-OST-1 and 3-OST-3, we tested the hypothesis for 3-OST activity. The plasmid expressing 3-OST-5 was transiently transfected into exponentially growing COS-7 cells. The cells were solubilized with detergent. HS sulfotransferase activity was determined by incubating with unlabeled HS and [^{35}S]PAPS as described under "Experimental Procedures". The resultant [^{35}S]HS was subjected to nitrous acid degradation at pH 1.5 followed by sodium borohydride reduction to prepare [^{35}S]disaccharides. This approach has been employed to successfully characterize the sulfation sites of 3-OST-2 and 3-OST-3 (8,13).

The [^{35}S]disaccharides were resolved on RPIP-HPLC, and the chromatograms are shown in Figure 3. Comparing the profiles of the degraded [^{35}S]HS that was modified by pcDNA3 transfected cell (Figure 3A), we found additional [^{35}S]disaccharides in the 3-OST-5 modified HS (Figure 3B). Coeluting with appropriate disaccharide standards on RPIP-HPLC, we identified that three of those [^{35}S]disaccharides were 3-*O*-sulfated disaccharides with the structures of IdoUA2S-AnMan3S (eluted at 38.5 min), GlcUA-AnMan3S6S (eluted at 55.5 min), and IdoUA2S-AnMan3S6S (eluted at 70.3 min). A small peak of ^{35}S-labeled disaccharide with a structure of IdoUA2S-AnMan6S was also observed (eluted at 59.6 min). The IdoUA2S-AnMan6S was observed in HS modified

with pcDNA3 transfected COS-7 cells (data not shown), although the level of this disaccharide varied between experiments. The presence of IdoUA2S-AnMan6S, which is a common disaccharide in HS, was unlikely to be associated with the activity of 3-OST-5. We also detected a minor [^{35}S]peak at 48 min. The identity of this [^{35}S]peak was unknown. Taken together, these results suggest that the expressed 3-OST-5 has the anticipated 3-OST activity. It is important to note that IdoUA2S-AnMan3S and IdoUA2S-AnMan3S6S are characteristic disaccharides of 3-OST-3 modified HS (13), whereas GlcUA-AnMan3S6S is a characteristic disaccharide of 3-OST-1 modified HS (12).

The activity of 3-OST-5 in assisting herpes simplex virus 1 (HSV-1) entry

A previous study demonstrated that 3-OST-3 modified HS serves as a receptor for herpes simplex virus-1 entry (13). Since we detected the disaccharides of 3-OST-3 modified HS, it appeared logical that 3-OST-5 modified HS might also generate an entry receptor for HSV-1. Therefore, we decided to test this possibility using an approach that was previously published (13). A recombinant β-galactosidase-expressing HSV-1 strain [HSV-1(KOS)gL86] was used for the entry assay. This recombinant virus expresses β-galactosidase from an insert in the viral genome immediately upon entry into cells. As shown in Figure 4A, the mock transfected CHO cells are resistant to HSV-1 entry (no dark cells) as previously reported (29). In contrast, a significant number of CHO cells transfected with pcDNA3.1-3OST5 were rendered susceptible (Figure 4B, dark cells) to the entry of HSV-1. We estimated that about 20% of the cells were susceptible to HSV-1 infection, which coincided with the transfection efficiency. In separate sets of experiments, we also found that 3-OST-5 modified HS does not generate receptors for

other alphaherpesviruses. The viruses examined for entry via 3-OST-5 modified HS included wild-type HSV-2, HSV-1 Rid mutants (24), bovine herpesvirus and pseudorabies virus (data not shown).

Furthermore, we examined whether 3-OST-5 modified HS bound to gD. The results showed that 3-OST-5 modified HS has about two-fold increase in the binding to gD compared to the control, suggesting that 3-OST-5 modified HS generates gD-binding sites (Table 1). We noted that the gD-binding percentage of 3-OST-5 modified HS (9.0%) was less than that of 3-OST-3 modified HS (23.1%). Such deviation is likely due to the fact the 3-OST-5 enzyme was in a mixture containing other HS sulfotransferases, whereas purified 3-OST-3A enzyme was employed to prepare 3-OST-3-modified HS. Taken together, our results suggest that 3-OST-5 is capable of assisting the entry of HSV-1 by generating a receptor for gD. In addition, it is apparent that the mechanism for 3-OST-5-assisted HSV-1 infection is very similar to what was previously characterized for 3-OST-3. However, we could not conclude whether or not the saccharide sequences of the gD-binding site in 3-OST-5-modified HS and the sequence in 3-OST-3A-modified HS were identical.

Demonstration of 3-OST-5 modified HS binding to AT

From the result of the disaccharide analysis of 3-OST-5 modified HS as described above, we found that 3-OST-5 modified HS also contains GlcUA-AnMan3S6S. This observation prompted us to determine whether or not 3-OST-5 modified HS bound to AT. The results for the binding of [^{35}S]HS to AT are shown in Table 1. As expected, 37.0 % of 3-OST-1 modified HS bound to AT, whereas only 1.4% of 3-OST-3 modified HS bound to AT. Those results were consistent with the previous reports (8,12).

Comparing the percentages of HS^{act} between 3-OST-5 modified HS and control, we found that the binding of 3-OST-5 modified HS to AT was 6.3-fold higher than the control sample. The result suggests that 3-OST-5 modified HS binds to AT. The conclusion was further strengthened by the results as presented below.

We also determined the HS^{act} conversion activity of the cell extract from 3-OST-5 transfected cells, as this assay was specifically designed to measure the enzymatic activity that generates anticoagulant HS (12). We incubated nonanticoagulant [^{35}S] HS with cell extract and unlabeled PAPS. The resultant [^{35}S]HS was subjected to AT/ConA-affinity gel. We found that about 4.8 % of the [^{35}S]HS bound to AT/ConA-affinity gel after incubation with 3-OST-5 transfected cell extract, while only 0.12% of the [^{35}S]HS bound to AT/ConA-affinity gel after incubation with the control cell extract (transfected with pcDNA3). Our result demonstrated that the HS^{act} conversion activity in 3-OST-5 transfected cells was elevated by 40-fold. The data was consistent with the conclusion that 3-OST-5 has the activity in synthesizing HS^{act}.

We compared the composition of the ^{35}S-labeled disaccharides from 3-OST-5 modified HS^{act} and from 3-OST-5 modified HS^{inact} (nonantithrombin binding HS). The HS^{inact} and HS^{act} were separated by the AT/ConA-affinity approach as described under "Experimental Procedures". Nearly 35% of 3-OST-5 modified HS^{act} bound to AT-affinity gel (Table 1), suggesting that the fractionation had significantly enriched HS^{act}. Both the HS^{inact} and HS^{act} were degraded with nitrous acid at pH 1.5 followed by sodium borohydride reduction, and the resultant [^{35}S]disaccharides were analyzed by RPIP-HPLC (Figure 5). As shown in Figure 5B, ^{35}S-labeled GlcUA-AnMan3S6S was the major disaccharide in the 3-OST-5-modified HS^{act}. It is noteworthy that GlcUA-

AnMan3S6S is a characteristic disaccharide of 3-OST-1 modified HS and is part of the AT-binding site (12). We also noted that GlcUA-AnMan3S6S was still observed in HS^{inact}. Two possible reasons might contribute to this observation. First, the AT-affinity fractionation was incomplete. Second, it is known that HS^{inact} contains GlcUA-AnMan3S6S (12). Taken together, our results demonstrated that 3-OST-5 enzyme generated an AT-binding site, and the HS^{act} contained the disaccharide, GlcUA-AnMan3S6S. In conclusion, our results indicated that 3-OST-5 synthesized both HS^{act} and gD-binding HS (8).

Of particular interesting is that two ^{35}S-labeled disaccharides, IdoUA2S-AnMan3S and IdoUA2S-AnMan3S6S, were absent in 3-OST-5 modified HS^{act} (Figure 5B). Those two disaccharides are believed to be parts of the gD-binding site in 3-OST-5 modified HS, which contribute to the activity in assisting HSV-1 entry. The data suggested that 3-OST-5 enzyme sulfates two subpopulations of HS substrates. One population is HS^{act} precursor, which becomes HS^{act} after 3-OST-5 modification. Another population is gD-binding HS precursor, which becomes gD-binding HS after 3-OST-5 modification. The data supported the conclusion from the previous reports, which demonstrated that the biosynthesis of HS^{act} is regulated by the availability of the HS^{act} precursors for 3-*O*-sulfation (23,30).

Tissue distribution of 3-OST-5

Northern analysis was carried out on a human northern-multiple tissue blot using 3-OST-5 open reading frame as a probe. It appears that 3-OST-5 is predominantly expressed in skeletal muscle with a size of ~2.4 Kb and ~3.8 Kb (Figure 6, top panel). The

distribution of 3-OST-5 is distinct from those of 3-OST-1 and 3-OST-3A and 3-OST-3B as reported by Shworak and colleagues(28).

DISCUSSION

HS is composed of sulfated glucosamine and glucuronic/iduronic acid residues. The 3-*O*-sulfated glucosamine is a rare constituent in the HS from natural sources, and plays critical roles in binding to AT (31), herpes simplex virus-1 envelope protein gD (13), growth factor receptor (32) and fibroblast growth factor 7(33). Although we know that the saccharide sequences of the gD-binding site and AT-binding sites are distinct (13), it still remains to be investigated whether or not the sequences for the bindings of FGF 7 and FGF receptor are identical to the AT-binding site. The 3-*O*-sulfated glucosamine residue is biosynthesized by 3-OST. At least five different isoforms of 3-OST isoforms have been identified (34). The characteristic disaccharides of the 3-OST-modified HS and the biological functions of the modified HS are summarized in Table 2.

In this communication, we report a new member of 3-OST family. From our data, we were unable to conclude that the ATG of 3-OST-5 represents the genuine initiation codon because we did not obtain further 5'-upstream cDNA sequence. It should be noted that 5'-untranslated regions play an important role in determining the translational efficiency of heparan sulfate *N*-deacetylase/*N*-sulfotransferase isoforms in different tissues (35). Nevertheless, the isolated 3-OST-5 contains a transmembrane domain, which predicts a type II-membrane bound protein, and a functional sulfotransferase domain. Those domains are found in the previously characterized full-length 3-OST-2 and 3-OST-3 (28), although the full-length 3-OST-1 lacks transmembrane domain(14). If

an additional sequence were present in 3-OST-5 coding region, we would expect that it would encode an additional cytosolic domain. It is known that the cytosolic domains of previously characterized 3-OST do not to contribute to the enzymatic activities[2] (11,14).

Amino acid homology search revealed that 3-OST-5 has 58% homology to 3-OST-3 and 72 % homology to 3-OST-1, respectively, in the predicted sulfotransferase domain. The data suggest that the amino acid sequence of 3-OST-5 is more close to 3-OST-1. However, we failed to identify a domain in 3-OST-5 that is specifically homologous to 3-OST-1 or to 3-OST-3, implying that the substrate specificities of 3-OST isoforms are determined by the three-dimensional structures of the enzymes. We noted that 3-OST-1 is a secreted protein as it lacks a transmembrane domain (14), whereas, 3-OST-5 is likely a membrane bound protein with a predicted transmembrane domain. Indeed, we failed to detect 3-OST-5 activity in the media[3]. A recent report demonstrated that the transmembrane domain of the 3-OST had no effects on their substrate specificities (36). Based upon their results, we concluded that the additional transmembrane domain of 3-OST-5 is unlikely to provide profound effect on the activities in synthesizing HS^{act} and gD-binding HS.

The substrates specificity of 3-OST-5 was characterized by determining the identities of the ^{35}S-labeled disaccharides from low-pH nitrous acid degraded 3-OST-5 modified HS. We found that the enzyme generates at least three 3-*O*-sulfated disaccharides: IdoUA2S-AnMan3S, GlcUA-AnMan3S6S, and IdoUA2S-AnMan3S6S[4]. Among these disaccharides, IdoUA2S-AnMan3S and IdoUA2S-AnMan3S6S are the characteristic disaccharides of 3-OST-3 modified HS, whereas, GlcUA-AnMan3S6S is a characteristic disaccharide of 3-OST-1 modified HS (11,12). Thus, we concluded that 3-

OST-5 possesses both the activities of 3-OST-1 and 3-OST-3. It is known that 3-OST-1 generates HS^{act}, whereas 3-OST-3 generates entry receptor for HSV-1(12,13). As expected, 3-OST-5 modified HS binds to AT and gD. In addition, transfection of the plasmid expressing 3-OST-5 rendered the susceptibility of HSV-1 to wild type CHO cells. Because both 3-*O*-sulfated glucosamine *N*-sulfate (GlcNS3S±6S) and 3-*O*-sulfated *N*-unsubstituted glucosamine ($GlcNH_23S±6S$) are susceptible to low-pH nitrous acid degradation (8), we could not conclude whether or not 3-OST-5 sulfates *N*-unsubstituted glucosamine or *N*-sulfated glucosamine. Such conclusion will become possible when a purified 3-OST-5 enzyme is available. Regardless of the limitations in the disaccharide analysis, our results indicate that 3-OST-5 has both 3-OST-1- and 3-OST-3-activities. It should be noted that two additional isoforms of 3-OST, 3-OST-2 and 3-OST-4, were reported (28). It is known that 3-OST-2 does not generate HS^{act} (8).

Our Northern analysis suggests that 3-OST-5 is predominantly expressed in the human skeletal muscle tissue. A previous report demonstrated that 3-OST-1 and 3-OST-3 (including 3-OST-3A and 3-OST-3B) were widely expressed in numerous human tissues, but low in skeletal muscle (28). The tissue distribution of 3-OST-5 suggests that 3-OST-5 modified HS may provide a unique biological function in this tissue. It is very interesting to note that a unique subset of HS was identified in human skeletal muscle tissues by a set of antibodies that bind to HS (37). An elegant study was recently published to determine the binding sequences of these antibodies (38). However, it still remains to be investigated whether or not the unique subset of HS found in the skeletal muscle tissue is associated with 3-OST-5 modification.

Clinical manifestations of herpes simplex virus are typically seen in two types of cells: mucosal epithelium with characteristic herpetic lesions, and in a few cases, neuronal cells, causing life-threatening encephalitis. Acute viremia exists but very rarely seen in infected patients. Based on the current knowledge, it is unlikely that infection of HSV in skeletal muscle has long-term pathogenic effects. It is very interesting to note that recent studies show that herpes simplex virus 1 can be utilized as a viral vector to deliver genes to the human skeletal muscle (39,40). For example, a specially engineered herpes simplex virus 1 was used for duchenne muscular dystrophy (39). The advantages for using herpes simplex virus 1 vector include high transduction efficiency and the capability to pack a large gene (40). Given the facts that 3-OST-5 modified HS serves as an entry receptor for HSV-1 and it is expressed in the human skeletal muscle, it is possible that the 3-OST-5 modified HS could serve as a receptor in skeletal muscle tissues for HSV-1 infection. If this is the case, further investigation of the mechanism for the 3-OST-5 assisted HSV-1 entry will be beneficial for not only understanding/modulating the pathogenic effects of the virus but also for its improved use as an important gene therapy vector for treating muscular dystrophy.

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Abbreviations

HS, heparan sulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; HSV-1, herpes simplex virus type 1; gD represents herpes envelope glycoprotein D; AT represents antithrombin; HS^{act} represents anticoagulant-active or antithrombin-binding HS; HS^{inact} represents nonantithrombin-binding HS; 3-OST: HS D-glucosaminyl 3-*O*-sulfotransferase; GlcUA, D-glucuronic acid; IdoUA, L-iduronic acid; IdoUA2S, L-iduronic acid 2-*O*-sulfate; AnMan, AnMan3S, AnMan6S, and AnMan3S6S represent 2,5-anhydromannitol, 2,5-anhydromannitol 3-*O*-sulfate, 6-*O*-sulfate, and 3,6-*O*-disulfate.

Footnotes

1. Human 3-OST-3A and 3-OST-3B have nearly identical amino acid sequence in the proposed sulfotransferase domain. Both enzymes sulfate identical disaccharides and have the activity in assisting HSV-1 entry. For the clarity, we use 3-OST-3 to represent both 3-OST-3A and 3-OST-3B in this article unless it is specified.
2. A truncated 3-OST-3A by removing its intracellular and transmembrane domains maintains its activity. 3-OST-1 enzyme that was originally purified from L cells media was a truncated form, which lacks the first 20 amino acid residues from the *N*-terminal.
3. 3-OST-1 is a secreted enzyme. The activity was readily detected in the media (14). The failure for detecting 3-OST-5 activity in the media also suggests that it was unlikely that the transfection of the plasmid expressing 3-OST-5 activates the expression of previously known 3-OST-1 in COS-7 cells.
4. We noted that 3-OST-1 modified HS also generates GlcUA-AnMan3S. We did not investigate whether 3-OST-5 generates GlcUA-AnMan3S because we utilized the RPIP-HPLC condition that is specifically designed to resolve disulfated or trisulfated disaccharides. Thus, we still do not know if 3-OST-5 generates GlcUA-AnMan3S.

Figure Legends

Figure 1. **The nucleotide and deduced amino acid sequences of 3-OST-5 (GenBank™ accession AF503292).** The single predicted membrane spanning domain and four

potential *N*-linked glycosylation sites are indicated by the double-underline and by the underlines with a • below the glycosylated Asn, respectively.

Figure 2. **Multiple amino acid sequence alignment of human 3-OST-5 with human 3-OST-1, 3-OST-3A and 3-OST-3B.** The alignment was performed by using the program BioEdit. Introduced gaps are shown as hyphens, and aligned amino acids are boxed and shaded with black for identical residuals and dark gray for similar residuals. 5'-PSB represents the putative domain that binds to 5'-phosphate of PAPS, and 3'-PSB represents the putative domain that binds to 3'-phosphate of PAPS.

Figure 3. **RPIP-HPLC chromatograms of the disaccharide analysis of 3-OST-5 modified HS.** Cell extracts from COS-7 cells transfected with the empty plasmid vector pcDNA 3 (*Panel A*) or with the plasmid expressing 3-OST-5 (*Panel B*) were incubated with unlabeled HS and [^{35}S]PAPS to prepare the [^{35}S]HS. The resultant [^{35}S]HS was depolymerized by nitrous acid at pH 1.5 followed by sodium borohydride reduction. The resultant [^{35}S]disaccharides were resolved on RPIP-HPLC. The elution positions of the disaccharide standards were indicated by arrows, where 1 represents IdoUA2S-AnMan3S; 2 represents GlcUA-AnMan3S6S; 3 represents IdoUA2S-AnMan6S; 4 represents IdoUA2S-AnMan3S6S.

Figure 4. **Entry of HSV-1 into CHO-K1 cells and transfected CHO-K1 cells.** CHO-K1 cells were transfected with control plasmid (A) or with pcDNA3.1-3OST5 (B). At 36 h after transfection, the cells were exposed to KOS-gL86 at 100 pfu/cell. Six hours later, the cells were washed, fixed, and incubated with X-gal to identify infected cells (dark cells).

Figure 5. **RPIP-HPLC chromatograms of the disaccharide analysis of 3-OST-5 modified HS^{inact} and HS^{act}.** HS^{act} was prepared from 3-OST-5 modified HS using an AT-affinity column. HS^{inact} was the fraction that does not bind to AT-affinity column. HS^{act} was eluted from the AT-affinity column with 1 M NaCl. Both HS^{inact} and HS^{act} were degraded with nitrous acid (pH 1.5) followed by sodium borohydride reduction. The arrows indicate the elution positions of disaccharide standards as described in Figure 3.

Figure 6. **Expression of 3-OST mRNA in human tissues.** A human multiple-tissue Northern blot was hybridized with human 3-OST-5 (upper panel) and ß-actin (lower panel) cDNA probes labeled with [^{32}P]dCTP under the conditions described in "Experimental Procedures"

Table 1. The binding of 3-OST-5 modified HS to gD and AT

	Binding to gD[1] (%)	Binding to AT [2] (%)
Control[3]	5.0 ± 0.4 (n=2)	0.68 ± 0.24 (n=4)
3-OST-5 modified HS	9.0 ± 0.1 (n=2)	4.3 ± 1.8 (n =4)
3-OST-5 modified HS^{act} [4]	Not determined	34.7 (n=1)
3-OST-1 modified HS[5]	6.6 ± 0.3 (n=2)	37.0 ± 3.3 (n =4)
3-OST-3 modified HS[5]	23.1± 3 (n=2)	1.4 ± 0.7 (n =4)

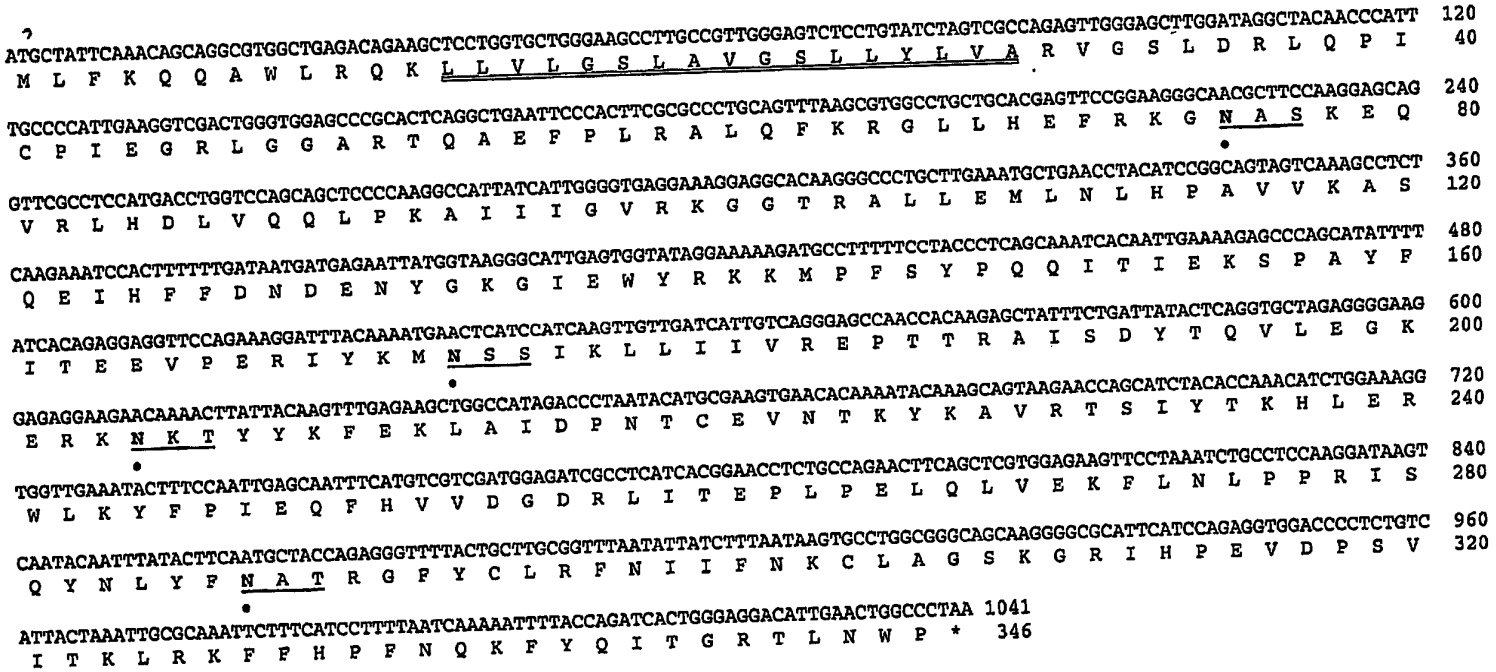
1. The binding of the HS and gD was determined by incubating modified [^{35}S]HS with gD followed by immunoprecipitation using anti-gD monoclonal antibody (DL6) to precipitate the complex of [^{35}S]HS and gD. Data are presented as the mean ± SD, where n represents the number of determinations.
2. The binding of the HS to AT was determined by incubating modified [^{35}S]HS and AT by using AT/ConA-Sepharose gel as described under "Experimental Procedures".
3. Control was the [^{35}S]HS that was prepared by incubating HS with the cell extract transfected with pcDNA3.
4. 3-OST-5 modified HS^{act} was prepared by AT-affinity fractionation from 3-OST-5 modified HS as described under "Experimental Procedures".
5. 3-OST-1 and 3-OST-3 modified HS were prepared by incubating unlabeled HS (from 33 cells), [^{35}S]PAPS and purified 3-OST-1 (70 ng) and 3-OST-3 (35 ng), respectively.

Table 2. Summary of the products and biological functions of 3-OST isoform modified HS[1]

3-OST isoforms	The characteristic disaccharides of enzyme modified HS[2]	Biological functions of the enzyme modified HS
3-OST-1	GlcUA-AnMan**3S**±6S[3]	AT-binding HS
3-OST-2	GlcUA2S-AnMan**3S** and IdoUA2S-AnMan**3S**	unknown[4]
3-OST-3A	IdoUA2S-AnMan**3S**±6S	Entry receptor for HSV-1
3-OST-3B	IdoUA2S-AnMan**3S**±6S	Entry receptor for HSV-1
3-OST-4	unknown	unknown[4]
3-OST-5	GlcUA-AnMan**3S**6S and IdoUA2S-AnMan**3S**±6S	AT-binding HS and Entry receptor for HSV-1

1. The information for 3-OST-1, 3-OST-2, 3-OST-3A and 3B was taken from Reference (34).
2. The disaccharides were prepared by subjecting the enzyme-modified HS to the degradations of nitrous acid.
3. The 3-*O*-sulfate group was bolded and underlined to emphasize the modification.
4. A recent review by Shukla and Spear indicated that 3-OST-2 and 3-OST-4 generate entry receptors for HSV-1(19).

Figure 1, Xia, G. *et.al.*



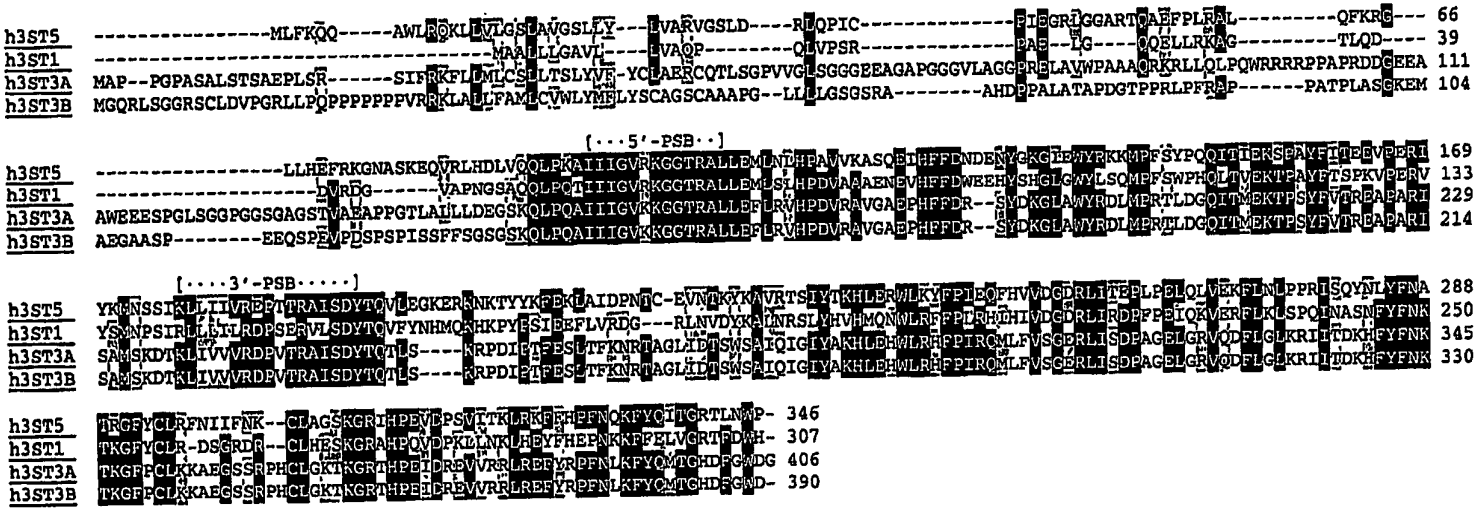
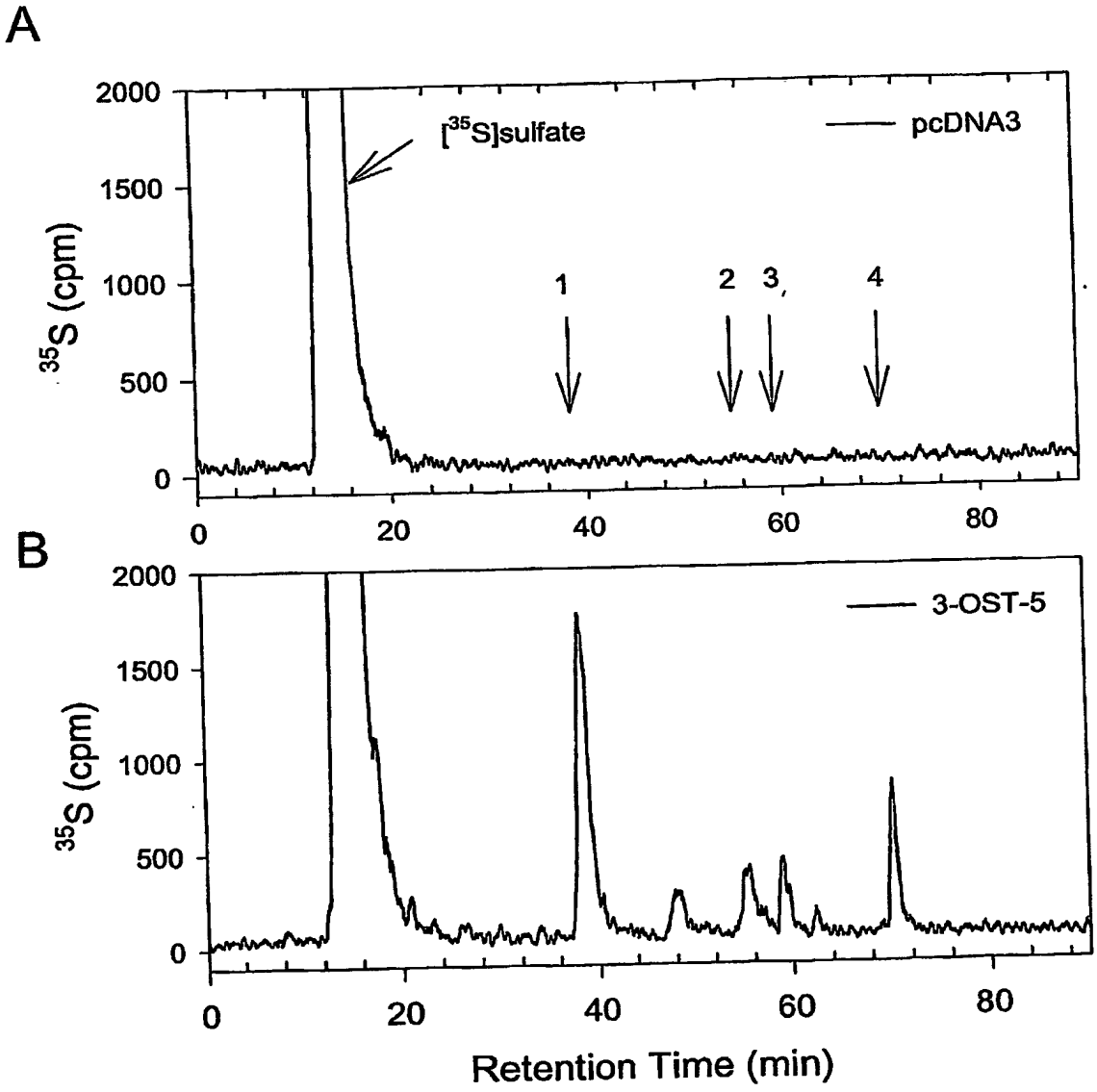
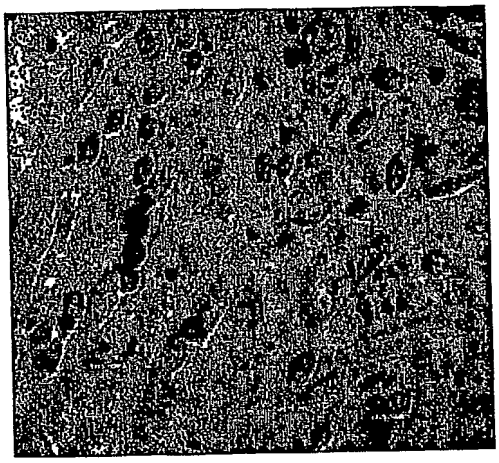


Figure 2 Xia, G. *et.al.*



Xia, G. et.al. Figure 3

A.



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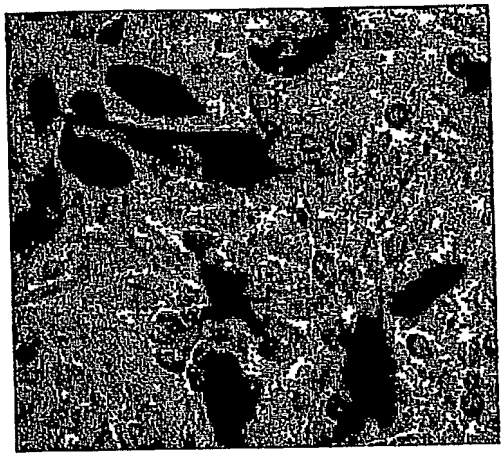
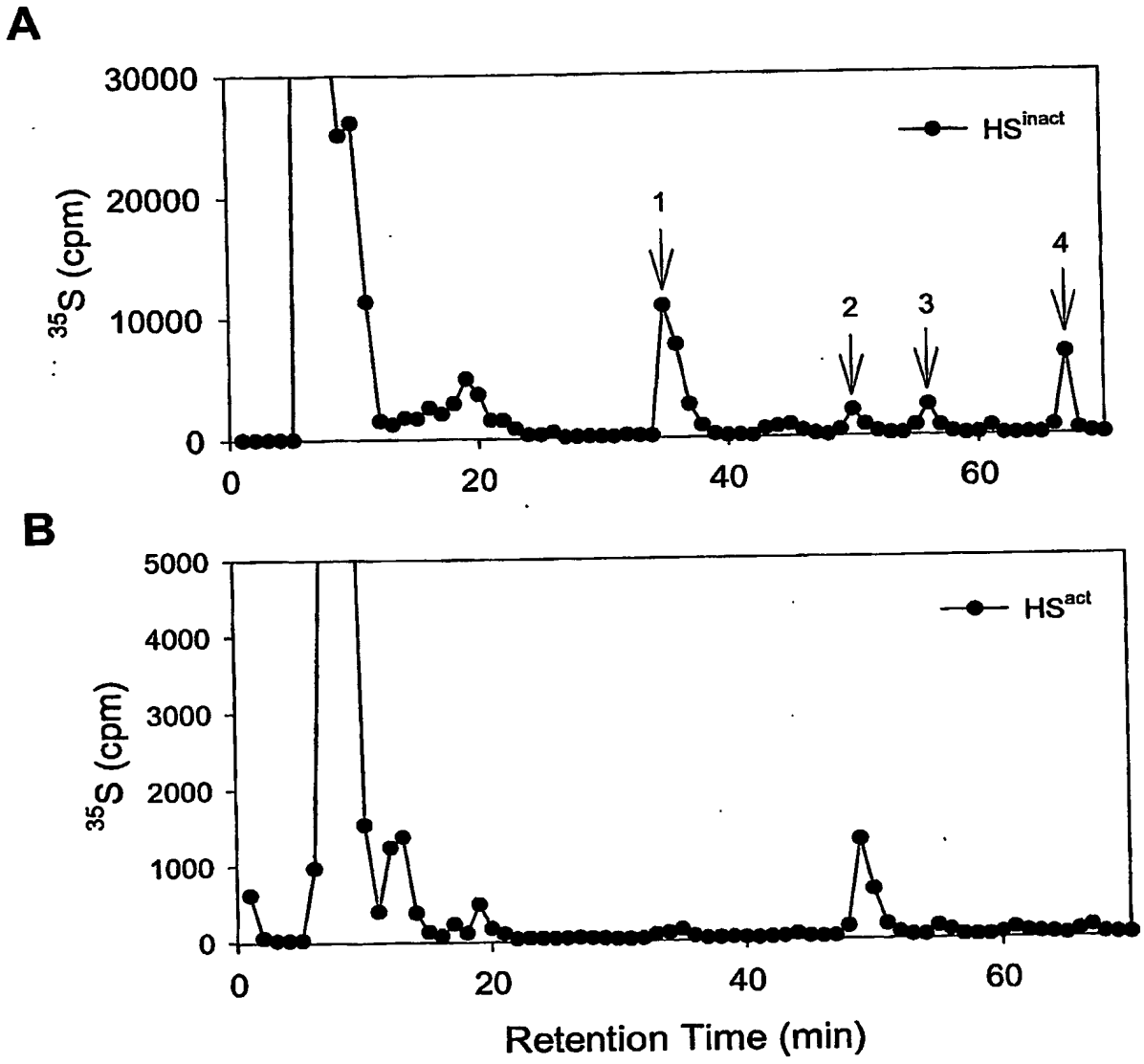
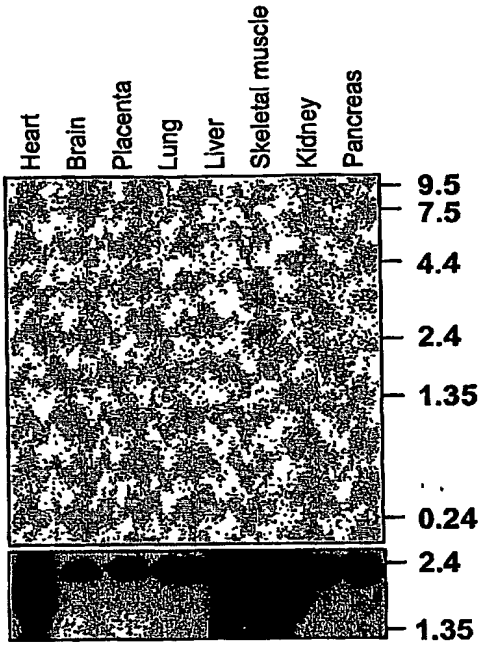


Figure 4, Xia, G. et. al.



Xia, G. et. al. Figure 5



Xia, G. et.al., Figure 6

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